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Using Genetic Markers to Identify Lung Cancer in Fine Needle Aspiration Samples

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Abstract

Purpose—We seek to establish a genetic test to identify lung cancer using cells obtained through CT guided fine needle aspiration (FNA).

Experimental Design—We selected regions of frequent copy number gains in chromosomes 1q32, 3q26, 5p15, and 8q24 in non-small cell lung cancer (NSCLC) and tested their ability to determine the neoplastic state of cells obtained by FNA using fluorescent *in situ* hybridization (FISH). Two sets of samples were included. The pilot set included six paraffin-embedded non-cancerous lung tissues and 33 formalin-fixed FNA specimens. These 39 samples were used to establish the optimal fixation and single scoring criteria for the samples. The test set included 40 FNA samples. The results of the genetic test were compared with the cytology, pathology, and clinical follow up for each case to assess the sensitivity and specificity of the genetic test.

Results—Non-tumor lung tissues had ≤4 signals per nuclei for all tested markers while tumor samples had ≥5 signals per nucleus in five or more cells for at least one marker. Among the 40 testing cases, 36 of 40 (90%) FNA samples were analyzable. Genetic analysis identified 15 cases as tumor

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and 21 as non-tumor. Clinical and pathological diagnoses confirmed the genetic test in 15 of 16 lung cancer cases regardless of tumor subtype, stage, or size and in 20 of 20 cases diagnosed as benign lung diseases.

Conclusions—A set of only four genetic markers can distinguish the neoplastic state of lung lesion using small samples obtained through CT guided FNA.

Keywords

FNA; lung cancer; genetic markers; chromosome amplification; in situ hybridization

Introduction

Lung cancer is a leading cause of cancer deaths worldwide. In year 2008, a total of 215,020 new lung cancer cases and 161,840 deaths are expected in the United States alone (1). The exceptionally high mortality rate of lung cancer is, in part, due to the fact that lung cancer is often diagnosed at a late stage (2) when the prognosis is nearly always poor. The ability to diagnose the disease at an early stage has the potential to save lives.

In deed, recent progress using computed tomography (CT) to screen high risk populations has demonstrated highly promising results for detecting lung cancer at an early stage when it is curable (3-6). Several studies have observed that 60-85% lung cancers detected by spiral CT are at stage I (7). However, a major concern in CT screening is the high incidence of finding suspicious nodules that are not cancer. Some studies have shown that 50% of the participants will have at least one noncalcified nodule (8). In CT studies, the number of patients who required further evaluation but did not have cancer, ranged from 5-50% in prevalence screening and 3-12% in incidence screening (9).

Outside of the screening scenario, patients with potentially suspicious nodules are often followed-up by CT guided FNA and pulmonary cytology in clinical practice (10). Diagnosis can be facilitated by clinical history along with laboratory and radiological findings, and it has recently been shown that cytological diagnosis is reliable for early lung cancer (11). However, the cytological differentiation of reactive pulmonary processes from malignant neoplasms can be challenging as reactive type 2 pneumocytes can be difficult to distinguish from malignant cells (12,13). In a retrospective analysis of FNA with or without core biopsies involving 95 cases that were identified as benign, 21 (22%) had specific benign diagnosis and all were true-negative for malignancy based on radiologic (n=17) or surgical (n=4) follow-up (14). The remaining 74 were either nonspecific benign (n = 53, 56%) or nondiagnostic (n = 21, 22%). Significantly, seven of the benign nonspecific (13%) and six of the nondiagnostic cases (29%) exhibited malignancy at excisional biopsy or radiologic follow-up. While this may be related to sampling error, it could also in part related to difficulties in interpretation based on cytology alone (15). A molecular test based on genetic changes frequently associated with lung cancer has the potential to help identify lung cancer in image-guided fine needle aspirates by providing tumor specific information.

Extensive genetic studies have demonstrated that chromosome copy number alterations, gene mutations, and gene expression changes are frequent events in tumor cells and are involved in lung cancer development. Comparative genomic hybridization (CGH) studies have identified recurrent chromosomal aberrations, particularly amplifications and deletions, in non-small cell lung carcinoma (NSCLC). In particular, gains at 1q31, 3q25-27, 5p13-14 and 8q23-24 and deletions of 3p21, 8p22, 9p21-22, 13q22, and 17p12-13 are frequently found in (16-20). In addition, 7p amplifications have been observed in a significant number of NSCLC (19-22) as well as in nontumorous lung specimens of some long term smokers (21).

In the present study, we selected a panel of four genetic markers that showed frequent gains in NSCLC based on CGH studies (19,20) and tested their ability to identify tumor in small biopsy sized samples. We first established a FISH based assay and developed evaluation criteria using non-tumor lung and lung cancer samples in a pilot study. We then tested the specificity and sensitivity of the genetic markers to identify lung cancer in a testing set of 40 routine FNA cytology samples with mixed tumor type and lung disease. Our results indicate that all lung cancers carry genetic amplification for the tested markers regardless of tumor stage, subtype, and size, while non-tumorous biopsies do not show any of these changes. We propose that the genetic test and markers that we developed here can be used to sensitively identify cancerous cells in small samples such as spiral CT-guided fine needle aspirates and other minimally invasive methods.

Materials and Methods

Tissue Samples

All samples were CT-guided fine needle aspirates (FNA) obtained using a 22 Gauge needle at the Weill Medical College of Cornell University following institutional-approved IRB (D. Y.). Cell aspirates were fixed directly in either formalin until analysis or prepared as paraffin cell blocks. Non-tumor lung samples (n =6) were obtained as paraffin sections discarded from the Armed Forces Institute of Pathology (AFIP). The genetic tests were performed on FNA cytology samples obtained at the time of the clinical evaluation when cytology diagnosis was made. Genetic test was done without knowledge or regard to the clinical diagnosis and was compared with clinical outcome after completion of the analysis. For suspicious nodules that underwent surgery, pathology reports and clinical follow-up was available on all cases. When nodules were benign, cytology and clinical follow-up was used. Outcome of genetic tests were compared with pathology/cytology and clinical follow-up to determine the reliability of the test. A total of 73 FNA cytology samples were used in this study and they included 33 small tumor FNA cytology samples preserved in formalin for the pilot tests, and 40 formalin-fixed (20 tumor and 20 benign) paraffin-embedded cell block sections for the genetic analysis. The final pathological diagnosis of the testing NSCLC samples included 12 adenocarcinoma (AD), three squamous cell carcinoma (SCC), four large cell carcinoma, three neuroendocrine tumors (NET), one small cell lung carcinoma (SCLC), and one poorly differentiated NSCLC. The non-tumor samples were diagnosed either as benign nonspecific (8) or benign-specific (12) based on cytology findings. In general, one to three sections of 10-30μm thick sections were used for the 40 paraffin-embedded test samples and one 10-50μm section was used for each of the six formalin-fixed, paraffin-embedded noncancerous lung samples. The patient information and tumors related information on the 40 test cases are shown in the Results.

Identification of Genetic Markers for Lung Cancer Detection

We selected candidate regions of genomic amplification based on two independent CGH studies involving lung adenocarcinomas (AD) and squamous cell carcinomas (SCC) (19,20). In total, we evaluated eight different chromosome arms frequently amplified in AD samples [1q, 3q, 5p, 7p, 8q, 12p, 20p, and X] and nine chromosome arms often amplified in SCC tumors [1q, 3q, 5p, 7p, 8q, 12p, 19q, 20p, and X]. An *in silico* test was then designed to determine the maximum number of lung cancers that could be identified based on the minimal number of genetic changes observed in these regions either alone or in combination in a set of 25 SSC and 59 AD cases (http://amba.charite.de/~ksch/cghdatabase/index.htm). The frequency of amplification for each marker was ranked based on the number of samples that could be detected for both AD and SC. Highest ranking markers for AD samples were cross tested with different high ranking markers for SC in different combinations to derive at a set of markers that jointly detected most cancers (Table 1).

Identification and Labeling of FISH Probes

Four to six overlapping bacterial artificial chromosomes (BAC) containing human chromosome regions identified via genetic analysis were selected for each region around the target genes (Centromere protein F [CENPF] for 1q31, TERC for 3q25-27, TERT for 5p13-14, and c-myc for 8q23-24) and obtained from public or commercial resources (BACPAC Resources, Children Hospital Oakland, CA and Invitrogen). DNA was isolated from each BAC; labeled with biotin-dUTP, and hybridized on normal blood lymphocytes metaphase-spread slides. Each BAC was evaluated for intensity and specific of hybridization at the target region, and a BAC contig comprising of two to four overlapping BAC clones was assembled for each region. These contigs served as probes for our genetic test in interphase nuclei by FISH. The probe for chromosome 8q was specific for the c-myc gene and was obtained from Vysis (cat # 32-190006). The resulting chromosome regions are shown in Table 1.

For probe, 2μg BAC DNA was labeled with biotin-dUTP or digoxigenin-dUTP by nick translation in the presence of 4nM labeled nucleotide. Approximately 100-200ng of labeled BAC probe was ethanol precipitated in the presence of 20μg each salmon sperm DNA and human Cot1 DNA. The dry pellet was dissolved in 5μL of hybridization buffer. The hybridization buffer was comprised of 50% deionized formamide, 10% dextran sulfate, and 1XSSC. The probe was denatured for 5 min. at 80°C and then preannealed for 1 h at 37°C before adding to the slides.

Sample Preparation and FISH Hybridization

To enable unambiguous signal evaluation, we prepared single nuclei suspensions from formalin-fixed paraffin-embedded cell block/tissue sections or from formalin-fixed tissues following the Hedley technique (23). Briefly, for paraffin-embedded cell/tissue, the sections were deparaffinized in xylene, hydrated through the ethanol series, and then incubated overnight in water at 4°C. For formalin fixed FNA cell aggregates, the formalin solution was removed and the cells were extensively washed with PBS to remove residual formalin and then incubated overnight in water at 4°C. The cells were digested with 0.1% protease Type XXIV (Sigma P-8038) in PBS at 45°C for 45-60 min and filtered through serum columns to remove undigested tissue. The purified single-cell suspensions were cyto-spun onto the slides. After air drying, slides were baked for 2 h at 60°C, dehydrated, and stored at 4°C in desiccators.

For hybridization, slides were incubated with sodium thiocyanate overnight at room temperature in a that varied from 0.1-0.5% for paraffin-embedded cells/tissue and 0.5-1.0% for samples that were formalin-fixed. After overnight incubation, slides were incubated in the same solution at 80°C for 1h and further treated with Zymed pretreatment (Tissue pretreatment kit; Zymed, CA) for 30 min at 95-98°C. After washing and dehydration, slides were denatured in 70% formamide and 2xSSC for 10 min at 80°C. The denatured slides were hybridized with preannealed probes, either individually or with another probe for 36 h at 37°C.

At the end of hybridization, the slides were washed in 50% formamide and 2xSSC at 45°C three times for 5 min. each, 0.5xSSC and 0.1%SDS at 65°C four times for 5 min. each and 2xSSC at room temperature. After washing, they were incubated with blocking buffer [4xSSC/0.1% Tween-20, 3% BSA] containing sheep or goat IgG (1:100 dilution) for 30 min at 37°C to block nonspecific binding. For biotin-labeled probes, the slides were incubated with 1:1000 dilution avidin-FITC (vector lab cat # A-2011) in developing buffer [4xSSC/0.1% Tween-20, 1%BSA] containing IgG for 1 h at 37°C. For digoxigenin-labeled probe, the slides were first incubated with 1:5000 dilution mouse anti-digoxigenin (Sigma cat # D8156) in a developing buffer containing IgG for 1 h at 37°C washed and then incubated with 1:1000 dilution of anti-mouse TRITC (Sigma cat # T2402) for 1 h at 37°C. For experiments where biotin and digoxigenin-labeled probes were used together, the slides were first incubated with mouse anti-

digoxigenin, and then with a combination of anti-mouse TRITC and Avidin-FITC each at a 1:1000 dilution. The slides were washed in 4XSSC-0.1% Tween-20 solution four times at 45° C to remove unbound label (FITC & TRITC). The detergent was removed by 2xSSC wash twice at room temperature and the slides were air dried and embedded in antifade solution containing DAPI.

Fluorescence Microscopy and FISH Scoring

All samples were analyzed using a Nikon E800 microscope under a 60x objective. The images were acquired using a Nikon E800 (Nikon Inc.) equipped with appropriate filters (Chroma Technologies) and acquired using an Retiga Exi digital camera (BioVision Technologies) at five to seven focal planes using the IPLab software.

The signals were evaluated by examine the entire slide without knowledge of the cytology, pathology or clinical follow up of the cases. Nuclei were examined individually to determine the number of signals per cells for each marker. Approximately 200 nuclei were scored for each sample/marker. Nuclei that could not be evaluated due to insufficient hybridization or cell clusters were excluded from scoring. The number of signals in the nuclei for each marker was counted until five or more signals were observed in three or more cells for at least one probe. When no amplification was observed in a particular sample, all four markers were evaluated and 130 or more nuclei per probe were counted. When amplification of five or more signals was detected for any one probe in more than five cells, the sample was considered a tumor regardless the status of the other markers. When samples were hybridized with two probes, the chromosome probes for 1q and 3q, and the probes for 5p and 8q (c-myc) were combined. In general, 200 nuclei were counted when enough cells were available. However, when cell numbers were limited in a particular sample, a minimum of 130 nuclei were counted. An independent observer (M.H.) was also scored samples without amplification.

Results

Detecting Lung Tumors Based on Chromosomal Amplification

We surveyed chromosome regions frequently amplified in AD and SCC tumors based on CGH findings (19,20). A database containing 25 SSC and 59 AD cases was then used to assess the ability of using genetic markers to identify lung cancers in an *in silico* test. As shown in Table 1, the number of lung cancers detectable varied from 75-80 samples (89.3-95.2%) when three different markers were used. When four markers (1q, 3q, 5p, and 8q) were used, 83 of 84 (98.8%) samples were detected (Table 1). The remaining AD sample did not have amplifications for any of the markers tested, and therefore, could not be detected by the test. Candidate genes that are frequently amplified in lung cancers in these four different chromosome regions were used as primary target of BAC probe selection.

To assess the specificity of the assay, we first tested six non-tumor lung tissues using the four selected probes. As shown in Fig. 1, 91-97% of all nuclei counted had two or less than two specific hybridization signals for each tested marker and 100% of the normal cells have four or less than four signals for all markers tested. The total number of nuclei counted ranged between 537-897 among the normal lung samples and 100% of the nuclei had four or less signals for each marker (Figs. 1, 2A, 2B). In contrast, of the 25 formalin-fixed FNA tumor samples that were analyzable, 22 of them displayed five or more signals in at least two of the tested markers and three had six or more signals in at least one marker. The number of cells having more than five signals per nucleus varied from 6 to 178 for at least one marker in the tested samples. The number of cells with two or less signals varied from 3-87% depending upon tumor content, with an average of 51.8% in the samples. Results for the 25 fully analyzed samples are summarized in Fig. 3 and detailed in Table S1. Based on this result, we considered

a sample as non-tumor when all four tested markers had four signals or less per nucleus for each probe and a sample as tumor when there were five or more hybridization signals per nucleus for any probe in more than five cells.

Optimal Sampling for FISH Analysis Using FNA Cytology Samples

To determine the optimal method for biopsy sample processing, we examined the robustness of FISH signals among biopsy samples preserved in a formalin fixative for a various length of time (3-18 months) in the test set of 33 FNA cytology samples. Overall, the hybridization signal was observed in 27 samples. Two cases were analyzable only for two of the four markers and these markers had four or less signals (data not shown). Optimal results were routinely obtained for samples that were fixed for 45 days or less. The hybridization results were much more variable for those samples stored in formalin more than 45 days. In these cases, even though sufficient nuclei were isolated from the remaining six samples, the hybridization signals were too weak for evaluation despite repeated effort. These six cases together with the two partially analyzable samples were excluded from analysis. In contrast, all six paraffin-embedded nontumorous lung samples generated robust signals by FISH hybridization (100%). Therefore, we used routine formalin-fixed, paraffin embedded sections for the genetic analysis by FISH.

Specificity of Cancer Detection Using Genetic Markers by FISH

We next used the criteria established above to examine 40 routine paraffin sections of FNA cell blocks (Table 2) to determine if FISH alone could identify the neoplastic status of the samples. After the completion of the FISH analysis, genetic assessments on the FNA cytology samples were compared with the cytology, histopathological diagnosis, and clinical follow-up of the patients (Table 2). Overall, 36 of 40 paraffin-embedded FNA cytology samples (90%) were analyzable by FISH. Four samples could not be analyzed due to the lack of nuclei after sample processing. Samples were analyzed for all four markers in most cases (30). In some cases, fewer markers were used when a conclusive diagnosis could be made for the case (three markers in one case, two markers in five cases). Examples of tumor and nontumor samples analyzed by FISH are shown in Figs. 2 and summarized in Fig. 3. In total, 15 cases had amplified signals for at least two markers and thus were considered as tumors based on the genetic test. Nineteen samples showed four or less than four signals per nuclei per marker for all tested markers and were considered as normal. Sample 21 was analyzable for only two markers but all cells analyzed had four or less signals per nucleus for the two tested markers. Furthermore, more than 92% of the nuclei (94 % for 1q32 and 92% for 3q26) in this sample had two or less hybridization signals per cell and this is the same pattern as in non-tumors where the hybridization signals of two or less for all four markers were observed in greater than 90% of the nuclei. In Sample 23, a total of 959 nuclei were counted for all four markers and only one nucleus had five signals for the 3q probe. Therefore, both these cases were considered as non-tumor based on scoring criteria established in the pilot test.

Comparison of Genetic Diagnosis to Pathology and Clinical Follow-up

When the genetic diagnosis by FISH was compared with available histopathological and clinical data, genetic markers accurately identified 15 of 16 tumors and all 20 clinically non-tumor samples, i.e., 35/36 analyzable cases (Summarized in Fig. 3). Sample 23 has been followed-up for more than 3.5 years and is clinically stable with no further disease complication further supports the molecular diagnosis for this sample as non-tumor. In Sample 40, the genetic analyses for all four markers were within normal range with 88.9-94% cells having signals two or less per nucleus for each marker. At CT guided FNA, Sample 40 was obtained from a complex part-solid mass with large areas of mucus plugging and inflammation in the initial cytology diagnosis. A second FNA analysis was recommended and used for the diagnosis of adenocarcinoma with brochioloalveolar features. Genetic analysis was done using cell block

samples prepared from the first FNA cytology sample. A second test using another tissue section of the same cell block revealed chromosome amplifications in three of four markers examined.

Discussion

Chromosomal aberrations occur frequently in different cancers including lung cancer. Extensive catalogs of recurrent abnormalities in a wide range of solid tumors have been compiled from cytogenetic (Mitelman Database of Chromosome Aberrations in Cancer) and CGH (24), studies and are available online. The data indicates that each tumor type displays a nonrandom recurrent pattern of chromosomal aberrations which can be used to distinguish between normal tissue and tumors (25,26). In contrast to DNA-based tests such as CGH and LOH analysis, which require the use of samples highly enriched for tumor cell content, FISH visually identifies the chromosomal aberrations on metaphase chromosome or interphase nuclei of the individual cells. This approach has been used to identify colorectal carcinogensis (27,28), bladder cancer (29), head neck (30), lung carcinoma (31), cervical cancer (32-34), and germ cell tumors (35).

The value of FISH analysis in the diagnosis of lung cancer has been explored with a specificity of 82-100% and sensitivity of 72-87% when two chromosome probes were used (36-38). In our study using FNA samples, we accurately identify all non-tumor cases (20/20, 100% specificity) using just four genetic markers. We were able to identify lung tumors based on the molecular status of the cells obtained by FNA in 15/16 testable cases (94%). The work presented here demonstrates the potential for a genetic based test performed synergistically to cytology to increase the specificity and sensitivity of the clinical diagnoses using small lung lesions. Furthermore, the genetic markers that we used (Table 1) to distinguish tumor from non-tumor in CT-guided FNA specimens coincide with the high resolution genomic profiles of human NSCLC identifying chromosome regions 1q 31, 3q25-27, 5p13-14, and 8q23-24 as the minimum common regions most often amplified in lung cancer (39). Our results support the notion that although chromosomal aberrations are associated with various cancers, they are absent or very rare in nontumorous cells (40). In contrast, tumors almost always contain genetic amplifications of chromosomes regardless of size, stage, and pathological subtype (37). These specific changes can be used as specific molecular markers.

Although highly specific, the genetic method described here is imperfect because it is limited by the cellular content of the tumor, the sampling precision of the FNA procedure, the presence of the genetic changes in the tumor sample, and the added cost as well as time for the analysis. In our study, 10% (4 of 40) of the FNA cytology samples could not be tested genetically due to the lack of sufficient cells. Furthermore, one of the 16 tumor samples (sample 40) required a second test by both cytology as well as genetic analysis for accurate diagnosis possibly due to the fact that the tumor contained large areas of mucus plugging and inflammation. Finally, although all testable tumor samples of this study had genetic changes in one or more makers, our initial survey using a larger set of 83 samples include one that would have missed by the genetic test. Nonetheless, our experience using routine formalin-fixed, paraffin-embedded cell block of FNA cytology samples showed that approximately 90% of the samples could be successfully analyzed by the genetic test using FISH. Of these analyzable samples, our genetic test identified all 20 (100%) benign cases and 15/16 (93.8%) tumors in the testing samples. Taken together, the FISH based genetic test had a sensitivity of 88% (35 of 40) and at a specificity of 97.5 % for all 40 cases.

In summary, we demonstrate here that genetic markers applied to spiral CT-guided FNA cytology samples is highly sensitive for the diagnosis of lung cancer and highly specific in its ability to exclude cancer within a given specimen. This approach should be particularly useful

in complementing cytology diagnosis of benign or nonspecific benign diseases, especially when there is radiological confirmation that the needle has been properly placed within the lesion. The use of the larger core biopsies could allow better sample acquisition and further increase its detection sensitivity in identifying tumor from small lung lesions. A larger prospective study is also needed to further validate the genetic test described here for it potential clinical application.

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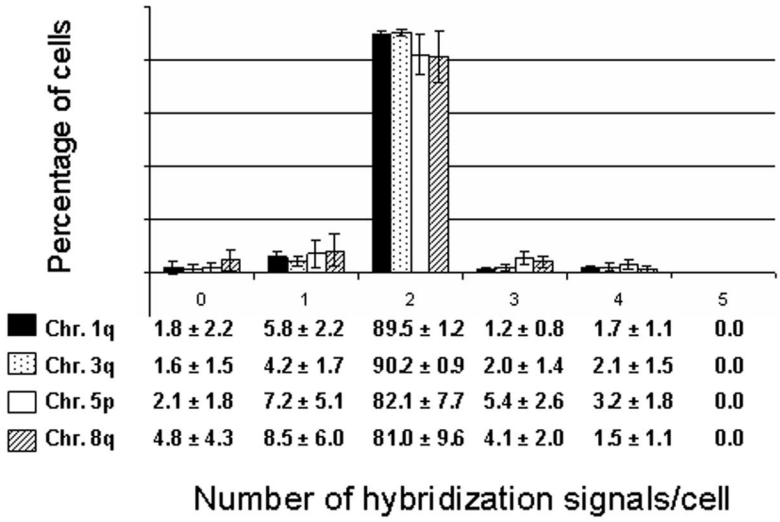


Figure 1.

Distribution of chromosome hybridization signals in noncancerous lung samples. Histograms represent the average percent of nuclei (y-axis) in six noncancer lung samples at indicated as 0 to 5 signal categories on the x-axis. The average percentage of each signal category ± SD for each marker is given in table underneath. The chromosome markers are shown by the pattern indicated at the left of the table.

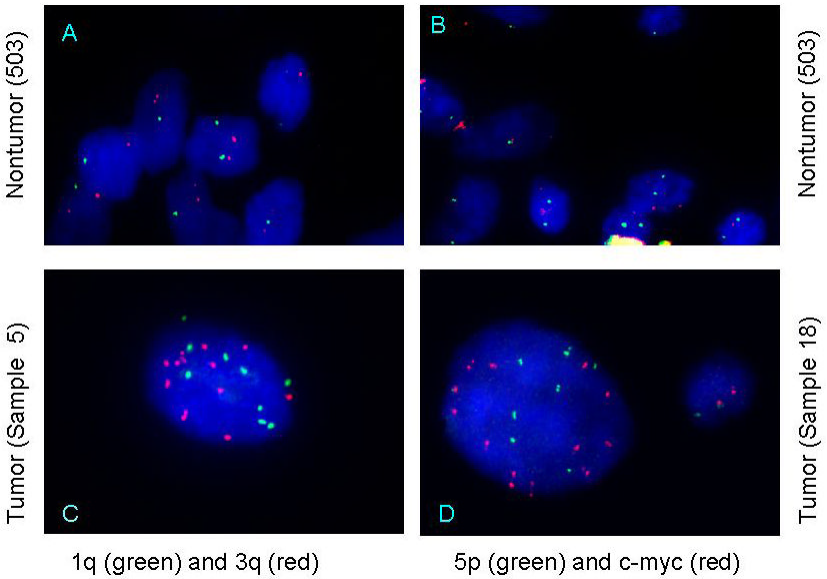
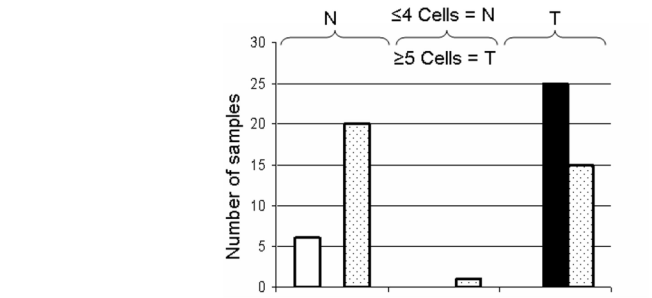


Figure 2.
In situ hybridization in tumor FNA and normal lung samples. A) and B) Non-tumor lung sample 503. C) and D) Tumor samples 14 and 18. Hybridization probes and colors are as indicated.



Samples		Maximum number of hybridization signals/cell ≤4	5	≥6	No. analyzable/Total
Training set	Normal	6	0	0	6/6
	FNA-Tumor	0	0	25	25/33
Test set	FNA-Biopsies	20	1	15	36/40

Figure 3.

Summary of FISH analysis on pilot and test samples. The samples were classified into three categories (≤4, 5, and ≥6) based on the observed maximum number of signals for each probe. Normal samples have a maximum number of ≤4 signals for each marker and tumors have a minimum number of ≥6 signals/marker or ≥5/marker in five or more cells.

- Normal: non-tumor lung samples
- FNA-pilot: tumors used in pilot test
- FNA-test: samples used in the testing analysis

Table 1

Detecting lung cancer based on chromosome copy number changes

Chromosomes with alterantion	Detectable based on any alteration (N=84)	Detecable (%)
1q, 3q, 5p, 8q	83	98.8
1q, 5p, 8q	80	95.2
1q, 3q, 8q	78	92.3
1q, 3q, 5p	77	91.7
3q, 5p, 8q	75	89.5

The lung tumor set included 25 squamous cell carcinomas and 59 adenocarcinomas and can be obtained from http://amba.charite.de/~ksch/cghdatabase/index.htm

Chromosome markers, 1q32, 3q26, 5p15, and 8q24 were used in combination or alone to determine the minimum number of markers that could detect the most tumors. Values show the number and the percentage of cancers detected for the indicated marker combinations.

Table 2

Clinical and genetic diagnoses of all 40 test FNA biopsies

Sample ID	Age	Gender	Cytology Diagnosis	Nodule Location	Pathology Diagnosis	Genetic Diagnosis	Agreement#
1	55	F	Atypical carcinoid	RUL	Carcinoid	Tumor	yes
2	78	F	Mucinous type adenocarcinoma	LUL	Adenocarcinoma	Tumor	yes
3	55	M	Well-differentiated adenocarcinoma	RLL	Adenocarcinoma	Tumor	yes
4	69	M	Adenocarcinom	LLL	Adenocarcinoma	Tumor	yes
5	62	M	Adenocarcinoma	LLL	Adenocarcinoma	Tumor	yes
6	55	M	Hamartoma	LLL	Benign	Normal	yes
7	45	M	High grade neuroendocrine carcinoma	RUL	Neuroendocrine	Tumor	yes
8	45	M	High grade neuroendocrine carcinoma	RUL	Neuroendocrine	Tumor	yes
9	83	F	Poorly differentiated non-small cell carcinoma	LLL	Non-small Cell carcinoma	NA	-
10	69	F	Non-small cell carcinoma	RLL	Non-small Cell carcinoma	Tumor	yes
11	65	M	Squamous cell carcinoma	RLL	Squamous cell carcinoma	Tumor	yes
12	60	F	Adenocarcinoma	LLL	Adenocarcinoma	Tumor	yes
13	NA	NA	Adenocarcinoma	hilum	Adenocarcinoma	NA	-
14	48	M	Adenocarcinoma	RUL	Adenocarcinoma	NA	-
15	64	M	Bronchioloalveolar carcinoma	RLL	Adenocarcinoma	Tumor	yes
16	60	M	Poorly differentiated squamous cell carcinoma	LUL	Squamous cell carcinoma	NA	-
17	78	F	Non-small cell carcinoma	LLL	Large cell carcinoma	Tumor	yes
18	70	M	Non-small cell carcinoma	RUL	Non-small Cell carcinoma	Tumor	yes
19	66	M	Small cell carcinoma	RUL	Small cell carcinoma	Tumor	yes
20	72	F	Adenocarcinoma	LUL	Adenocarcinoma	Tumor	yes
21	85	M	Bronchioloalveolar cell hyerplasia	LLL	Benign	Normal	yes
22	67	F	Acute inflammation	RML	Benign	Normal	yes
23	36	M	Acute and chronic inflammation	RUL	Benign	Normal	yes
24	36	M	Acute and chronic inflammation	LUL	Benign	Normal	yes
25	69	M	Atelectasis/scarring	RUL	Benign	Normal	yes
26	54	M	Atelectasis/scarring	RLL	Benign	Normal	yes
27	72	F	Atelectasis/scarring	lingular mass	Benign	Normal	yes
28	69	F	Inflammatory pseudotumor	para-arotic mass	Benign	Normal	yes
29	56	F	Pseuomonas aeruginosa	RUL	Benign	Normal	yes
30	43	M	Chronic inflammation with caseating granulomatous features	RUL	Benign	Normal	yes
31	55	M	Pulmunary hamartoma	RLL	Benign	Normal	yes
32	55	F	Fibromyxoid stroma	RUL	Benign	Normal	yes
33	75	M	Acute inflammation and necrosis with fungus forms present	RUL	Benign	Normal	yes
34	51	M	Acute pneumonia with bacilli	RUL	Benign	Normal	yes
35	89	M	Acute pneumonia with bacilli	RUL	Benign	Normal	yes
36	77	M	Acute inflammation with few dysplastic squamous cells	LUL	Benign	Normal	yes
37	75	M	Reative intrapulmonary lymph node	RLL	Benign	Normal	yes
38	34	M	Fibrous scar with elastosis and chronic inflammation	LLL	Benign	Normal	yes
39	58	F	Chronic inflammation	RML	Benign	Normal	yes
40	84	M	Fibrinous pneumonia	LUL	Adenocarcinoma	Tumor	No *

* confirmed it to be tumor in repeat test.